# THE BINDING SITE FOR THE 3′-TERMINUS OF AMINOACYL-tRNA IN THE MOLECULE OF ELONGATION FACTOR $T_u$ FROM *ESCHERICHIA COLI*

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## 1. Introduction

The mRNA-directed binding of aminoacyl-tRNA to the ribosome requires a special protein factor EF-$T_u$ and GTP. A ternary complex between aminoacyl-tRNA, EF-$T_u$ and GTP is formed as an intermediate from which the aminoacyl-tRNA is transferred to the ribosomal recognition site (reviewed [1]). It is the main feature of EF-$T_u$ that in the form of EF-$T_u$·GTP it is able to discriminate between aminoacylated and non- or acylaminoacylated tRNAs [2–4]. This provided a basis for the accumulating evidence that the 3′-terminus of aminoacyl-tRNA is involved in the recognition reaction between aminoacyl-tRNA and the elongation factor $T_u$ [5–7]. Therefore, we prepared two analogs of the 3′-terminus of phenylalanyl-tRNA, 2′(3′)-*O*-L-phenylalanyladenosine and cytidylyl-(3′ → 5′)-2′(3′)-*O*-L-phenylalanyladenosine and tested them for the ability to replace aminoacyl-tRNA in protecting the aminoacyl-tRNA binding site of EF-$T_u$ from inactivation by *N*-tosyl-L-phenylalanyl-chloromethane.

The results presented here show that:

(1) A-Phe and CpA-Phe can interact with the aminoacyl-tRNA binding site of EF-$T_u$;
(2) The SH group in the aminoacyl-tRNA binding area of EF-$T_u$ might be the component involved in the interaction between elongation factor $T_u$ and the 3′-terminus of aminoacyl-tRNA.

*Abbreviations:* A-Phe, 2′(3′)-*O*-L-phenylalanyladenosine; CpA-Phe, cytidylyl-(3′ → 5′)-2′(3′)-*O*-L-phenylalanyladenosine; TPCK, *N*-tosyl-L-phenylalanyl chloromethane, L-1-tosylamido-2-phenylethyl chloromethyl ketone

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## 2. Materials and methods

Crystalline EF-$T_u$·GDP was prepared according to [8]. tRNA from *E. coli* B was prepared by the method in [9]. Aminoacyl-tRNA: tRNA was charged with 8 L-amino acids (Leu, Val, Ala, Arg, Tyr, Glu, Ser, Gly) by the procedure in [10] except that each amino acid in the charging mixture was 0.2 mM. $^{14}$C-Labelled TPCK of spec. act. 4.6 mCi/mmol was obtained from the Laboratory for Radioactive Isotopes, Czechoslovak Academy of Sciences, Prague. 2′(3′)-*O*-L-phenylalanyladenosine was prepared by the method in [11] with some modifications of the purification step. The synthesis of cytidylyl-(3′ → 5′)-2′(3′)-*O*-L-phenylalanyladenosine will be described elsewhere.

Preparation of EF-$T_u$·GTP: 20 nmol crystalline EF-$T_u$·GDP was washed with 0.4 ml solution containing 42% saturated ammonium sulphate, 20 mM Tris–HCl (pH 7.6), 3 mM magnesium acetate and 5 mM 2-mercaptoethanol and 3 times with 0.35 ml of the same solution but with 10 μM GDP and without 2-mercaptoethanol. The sediment of crystals was dissolved in 0.4 ml buffer containing 100 mM Tris–HCl (pH 7.6), 10 mM magnesium acetate and 100 mM ammonium acetate. 5 μl 20 mM GTP, 20 μl 0.1 M phosphoenolpyruvate and 10 μg pyruvate kinase were added and the solution was incubated for 10 min at 35°C.

Detection of interaction between EF-$T_u$·GTP and aminoacyl-tRNA or the analogs of the 3′-terminus of aminoacyl-tRNA. The method is based on the ability of the compound TPCK to compete specifically with natural ligands for the aminoacyl-tRNA binding site in EF-$T_u$ [12–16]. 6 nmol EF-$T_u$·GTP (freshly prepared, in 130 μl buffer solution as above) either alone or in the presence of tested compounds (concentrations of A-Phe and CpA-Phe corresponded to the actual amount of aminoacylated compound) were treated with 10 nmol [$^{14}$C]TPCK in final vol. 155 μl, if not otherwise specified. The reaction was started by the addition of [$^{14}$C]TPCK (a methanolic solution of TPCK was used and the final concentration of methanol in the reaction mixture was ~6.5%) and carried out at 4°C. At the time intervals indicated, 20 μl portions from every incubation mixture were withdrawn and mixed with 5 μl methanol and 10 μl 0.1 M dithiothreitol for 3 min at 4°C to remove the free unreacted TPCK [10]. 10 μl (10 mg/ml) bovine serum albumin and 3 ml cold 5% trichloroacetic acid were further added and after 20 min at 4°C, the TPCK incorporation into EF-$T_u$ was determined as the radioactivity retained in the trichloroacetic precipitate collected on Millipore nitrocellulose filters which were washed with 10 ml cold 5% trichloroacetic acid and dried. The radioactivity was measured in a methane-flow windowless Frieseke-Hoepfner counter. 3100 cpm correspond to 1 nmol TPCK incorporated. Blank samples containing the same components except EF-$T_u$·GTP were treated in the same way as the whole systems and run simultaneously. The radioactivity retained in Millipore filters under these conditions (300–400 cpm) was subtracted from the values obtained for the complete systems. The protein concentration was determined by the method in [17].

## 3. Results

We employed an irreversible inhibitor of elongation factor $T_u$, *N*-tosyl-L-phenylalanyl chloromethane [18,19] to detect the interaction of the factor with the respective ligands for its aminoacyl-tRNA binding site. TPCK destroys the ability of EF-$T_u$·GTP to form the ternary complex with aminoacyl-tRNA [13,14] because it reacts specifically with the SH group of EF-$T_u$ which is essential for the interaction of the factor with aminoacyl-tRNA [15,20,21]. On the other hand, it is possible to show with the radioactively-labelled TPCK that a prior binding of aminoacyl-tRNA to EF-$T_u$·GTP protects the protein from TPCK incorporation (fig.1; cf. [15,16]) and this suggests that both components, i.e., aminoacyl-tRNA and TPCK, compete for the same binding site in EF-$T_u$. Deaminoacylated tRNA, which is not able to form a complex with EF-$T_u$·GTP, also has no effect on the incorporation of TPCK (fig.1), thus illustrating the specificity of the competition reaction.

If the 3′-end of aminoacyl-tRNA is involved in the binding reaction with EF-$T_u$, then it could be expected that this portion of the molecule of aminoacyl-tRNA alone should protect the factor from TPCK incorporation. Therefore, we prepared A-Phe and CpA-Phe as simple analogs of the 3′-end of aminoacyl-tRNA and tested them for the ability to prevent the reaction between TPCK and EF-$T_u$ (fig.2, table 1). It is evident that both CpA-Phe and even A-Phe protect EF-$T_u$·GTP against TPCK incorporation as does the whole molecule of aminoacyl-tRNA. In contrast, adenosine or an equimolar mixture of adenosine and phenylalanine (at 1 mM, data not shown) do not prevent the reaction between the factor and TPCK. Puromycin, which possesses the aminoacyl residue linked to the dimethyladenosine moiety of the molecule by an amide bond, instead of a natural ester bond, does not affect the

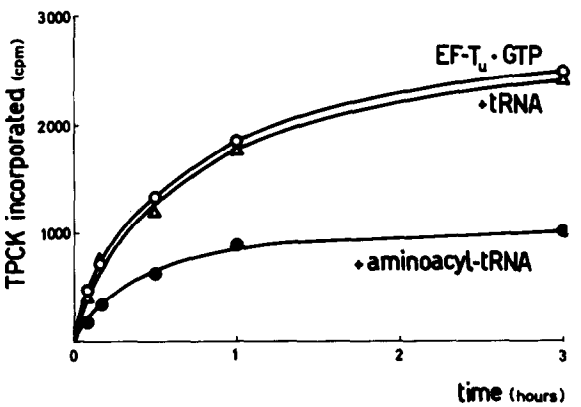


Fig.1. Protection of EF-$T_u$·GTP against TPCK incorporation by aminoacylated tRNA. Detection of interaction between EF-$T_u$·GTP and aminoacyl-tRNA was determined as in section 2. (○) EF-$T_u$·GTP, (△) EF-$T_u$·GTP + tRNA (160 μg), (●) EF-$T_u$·GTP + aminoacyl-tRNA (160 μg).

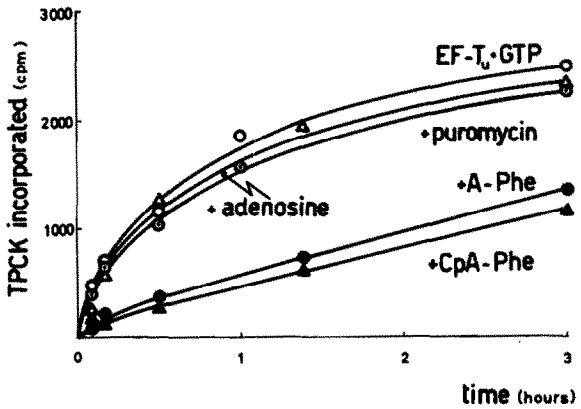


Fig.2. Protection of EF-$T_u$·GTP against TPCK incorporation by A-Phe and CpA-Phe. Detection of interaction between EF-$T_u$·GTP and the analogs of the 3′-terminus of aminoacyl-tRNA was determined as in section 2. (○) EF-$T_u$·GTP, (△) EF-$T_u$·GTP + 3.4 mM adenosine, (◎) EF-$T_u$·GTP + 1 mM puromycin, (●) EF-$T_u$·GTP + 3.4 mM A-Phe, (▲) EF-$T_u$·GTP + 1 mM CpA-Phe.

rate of incorporation of TPCK into EF-$T_u$·GTP either (fig.2).

From a comparison of the results presented in fig.2 and table 1 it is obvious that phenylalanyldinucleoside phosphate is more effective in protection of EF-$T_u$ than simpler phenylalanyladenosine. Under the experimental conditions employed, 50% shielding of EF-$T_u$·GTP against TPCK is observed with about $2 \times 10^{-4}$ M CpA-Phe or $8 \times 10^{-4}$ M A-Phe (table 1).

Table 1
Interaction of A-Phe and CpA-Phe with EF-$T_u$·GTP

| Addition to EF-$T_u$·GTP | | % TPCK incorp. |
|---|---|---|
| None | | 100 |
| Ado | ($10^{-3}$ M) | 94 |
| A-Phe | ($4 \times 10^{-4}$ M) | 65 |
| | ($8 \times 10^{-4}$ M) | 54 |
| | ($1.6 \times 10^{-3}$ M) | 35 |
| CpA-Phe | ($8 \times 10^{-5}$ M) | 75 |
| | ($4 \times 10^{-4}$ M) | 28 |
| | ($8 \times 10^{-4}$ M) | 20 |

1.2 nmol EF-$T_u$·GTP (in 20 μl buffer solution prepared as in section 2), either alone or in the presence of adenosine or A-Phe or CpA-Phe (at concentrations indicated) were treated with 2.1 nmol [$^{14}$C]TPCK in methanol in final vol 25 μl. After 30 min at 4°C, dithiothreitol and methanol were added to stop the reaction, and TPCK incorporation was determined as in section 2. 100% incorporation of TPCK corresponds to 1420 cpm

## 4. Discussion

In [15,16], we have shown that the compound, *N*-tosyl-L-phenylalanyl chloromethane, can be used as a site-directed inhibitor of the elongation factor $T_u$ for the specific labelling in its aminoacyl-tRNA binding site. Aminoacyl-tRNA, the natural ligand of EF-$T_u$·GTP whose binding to the factor is prevented by the inhibitor, was in turn found to protect the protein from incorporation of the radioactive TPCK. We tried to determine which part of the molecule of aminoacyl-tRNA was responsible for this activity, i.e., could participate in the interaction with EF-$T_u$. The findings that only the aminoacylated in contrast to deaminoacylated tRNA was active in the protection of EF-$T_u$·GTP against TPCK and other evidence [2–7] strongly suggested that especially the 3′-terminus of aminoacyl-tRNA could have been involved. To test this idea, we used two fragments of the 3′-end of phenylalanyl-tRNA, CpA-Phe and A-Phe, as analogs of this end of aminoacyl-tRNA and assayed them in our system with TPCK.

As shown in section 3, both tested fragments protect the elongation factor from incorporation of the inhibitor as does the whole molecule of aminoacyl-tRNA. This indicates that A-Phe and CpA-Phe can interact with EF-$T_u$·GTP.

The presence of an ester bond between an amino acid and adenosine seems to be essential for the protective ability of the tested analogs because adenosine alone or in conjunction with phenylalanine does not affect the incorporation of TPCK into the factor. Similarly, puromycin which resembles very closely phenylalanyladenosine but whose ester bond is replaced by an amide bond does not produce any shielding effect against TPCK (fig.2). All these findings show that the structure of the 3′-end of aminoacyl-tRNA is checked by EF-$T_u$·GTP with great efficiency. Further, it appears that the presence of the whole polynucleotide molecule is not necessary for the recognition reaction between EF-$T_u$ and aminoacyl-tRNA because the observed discrimination specificity of EF-$T_u$ against all 4 tested fragments of the 3′-termi-

nus of aminoacyl-tRNA parallels the known discrimination ability of EF-$T_u$·GTP against the structure of this area in the intact molecule of aminoacyl-tRNA. We have shown here that neither adenosine alone, just as deaminoacylated tRNA [2–4], nor puromycin, just as aminoacyl-tRNA in which the ester bond normally linking the amino acid to tRNA is replaced by an amide bond [7], are sufficient ligands for the aminoacyl-tRNA binding site in EF-$T_u$.

The results on protection of EF-$T_u$ against TPCK are in complete agreement with what we know of the requirements of the binding reaction between EF-$T_u$ and aminoacyl-tRNA. This suggests that the assay with the radioactively-labelled TPCK may be alternatively used as a specific and very simple system for the detection of interaction of EF-$T_u$ with aminoacyl-tRNA and with ligands for its aminoacyl-tRNA binding site.

The present results have also confirmed the findings [5] that cytidylyl phenylalanyladenosine can interact with EF-$T_u$. Moreover, we were able to show that even 2′(3′)-*O*-L-phenylalanyladenosine is active. Thus, A-Phe appears to be the smallest known ligand for the aminoacyl-tRNA binding site of EF-$T_u$ and the attachment of cytidylic acid residue to its 5′-end increases its affinity for the factor. This supports the general view that the nucleotide units at the 3′-terminus of tRNA are somehow intimately involved in the recognition and binding process between aminoacyl-tRNA and EF-$T_u$ [6] and gives evidence for the 3′-terminal aminoacyladenosine as being one of the components participating in this reaction. In this respect, the situation is reminiscent of another aminoacyl-tRNA binding site, the acceptor binding site on the ribosome. In contrast to the ribosomal site, however, EF-$T_u$·GTP discriminates between phenylalanyladenosine and puromycin.

Finally, these results provide, for the first time, the information on the localization of the binding site for the functionally important 3′-terminus of aminoacyl-tRNA in the molecule of EF-$T_u$. We have shown that A-Phe and CpA-Phe prevent the reaction between TPCK and the SH group of EF-$T_u$ essential for the binding with aminoacyl-tRNA. It can, therefore, be concluded that the SH group in the aminoacyl-tRNA binding area of the factor might be involved in the interaction between elongation factor $T_u$ and the 3′-terminus of aminoacyl-tRNA.

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